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Molecular typing of *Clostridium perfringens* isolated from minced meat

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Abstract

The aim of this study was to determine the presence of toxin genes in *Clostridium perfringens* isolated from 200minced meat samples using molecular typing. For this purpose, after isolation of *Clostridium perfringens* using conventional culture method and confirmation by specific 16Srd NA gene PCR, alpha (*cpa*), beta (*cpb*), beta 2 (*cpb2*),epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) genes were investigated by multiplex PCR.Out of 200 minced meat samples, 25 (12.5%) were determined as contaminated with *C. perfringens* and m-PCR results demonstrated that, out of these 25 isolates, 18 (81 %) possessed only the *cpa* gene (type A), while 4 (18 %) carried the *cpa* and *cpb2* toxin genes (type A-*cpb2*$^{+}$). Furthermore, the results showed1 isolate (4 %) as type B, 1 isolate (4 %) as type D and 1 isolate (4 %) as type E. This study reports the first *cpb2* positive type B of *C. perfringens* in minced meat. Our results also indicate that *C. perfringens* type A is the most common type in minced meat.

Keywords: *Clostridium perfringens*, toxin genes, minced meat

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Introduction

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic rod and ubiquitous pathogen, responsible fordifferent diseases such as gas gangrene, food poisoningand diarrhea in humans as well as for enterotoxemia and haemorrhagic gastroenteritis in many domestic and wild animals (Daube *et al*., 1994, Songer, 1996). The pathogenicity of the organism is associated with several toxins which are used for toxin typing of the bacteria. The alpha (*α*), beta (*β*), epsilon (*ε*) and iota (*i*) toxinsare the major lethal toxins produced by the organism that are closely related to its virulence, even though they produce several minor extra cellular toxins (Hatheway, 1990). *C. perfringens* is classified into five types (A–E) on the basis of their ability to produce major lethal toxins. Within these five types, all *C. perfringens* produce alpha toxin. In addition, type B strains produce *b* and *ε* toxins, type C produces *b* toxin, type D produces *ε* toxin and type E produces *i*toxin (Baums *et al*., 2004). In addition to the major lethaltoxins, a minority of *C. perfringens* strains produce anenterotoxin (*cpe*), which is responsible for the symptoms of common *C. perfringens* type A food poisoning (Erol *et al*., 2008). Different meats and meat products have been frequently reported as the most common food vehicles (Hatheway, 1990, Ridell *et al*., 1998, Hatakka and Halonen, 2000, Eisgruber and Hauner, 2001, McClane, 2001). About 2–5% of all *C. perfringens* isolates, mostly belonging to type A, produce *cpe*, a 35-kDa single polypeptide (Songer and Meer, 1996). *Cpe* positive *C. perfringens* type A is one of the most commonly reported food-borne pathogen in the US, EU and Turkey (McClane, 2001, Cakmak *et al.,* 2006, Hughes *et al*., 2007). Thus, detection of *C. perfringens* toxin types and subtypes iscritical for a better understanding of the epidemiology of *C. perfringens* infections and may be helpful in the developmentof effective preventive measures.

The classification of *C. perfringens* isolates into toxigenictypes has been traditionally performed by sero-neutralizationwith mice or guinea pigs (Oakley and Wayrack, 1953, Sterneand Batty, 1975; McDonel, 1986). Because these methods are time consuming and expensive, they have largely been replaced by PCR-based detection methods.Various PCR protocols, including multiplex PCR assay, have been established for genotyping of *C. perfringens* isolates with respect to the *cpa, cpb, etx, iap, cpe* and *cpb2* genes, encoding the *α, β, ε, i,*entero and *b2* toxins, respectively (Daube *et al*., 1994, Songer and Meer, 1996, Meer and Songer, 1997, Yoo *et al*., 1997, Kanakaraj *et al*., 1998, Kadra *et al*., 1999, Augustynowicz *et al*., 2000, Garmory *et al*., 2000).

However, there are no published data on molecular typing of *C. perfringens* in minced meat presented inretail markets and butcheries in Iran. In present study, the multiplex PCR assay was used in order to determine the presence of alpha (*cpa*), beta (*cpb*)*,* beta 2(*cpb2*), epsilon (*etx*), iota (*iA*) and enterotoxin (*cpe*) genes inisolated *C. perfringens* from minced meat samples.

Materials and methods

Bacterial isolation: Over three months period (fall) total of200 minced meat samples (10 g of mixed beef and sheep meat) were collected aseptically from different retail markets and butcheries in Mashhadcity.Samples were placed into a 50-ml sterile plastic tube for transfer to the laboratory. Upon arriving to the laboratory, processing of each meat sample started with homogenization step. Ten milliliters of sterile fluid thioglycolate (FTG) medium was then added to the 50-ml tube containing 10 g of the homogenated mincedmeat. Analiquot (1 ml) of each FTG meat suspension was added to each of two tubes containing 10 ml of sterile FTG medium. To enrich for any *C. perfringens*spores in the meat samples, one of those two tubes was heat shocked at 72°C for 20 minutes before

incubation at 37°C for 18 to 24 h. The other tube was directly incubatedat 37°C for 18 to 24h to enrich primarily for *C. perfringens* vegetative cells presentedin meat samples (Wen and McClane, 2004).

Each FTG enrichment culture was streaked onto nutrient bloodagar containing 7% defibrinated sheep blood and incubated anaerobically at 37ºC for 48 hr. Colonies which showed characteristic dual hemolytic zones were picked up and sub-cultured in TryptoseSulfiteCycloserine agar (TSC) and Tryptose Sulfite Neomycin agar (TSN) for purification. The identity of the isolates was confirmed by their colonial and microscopical morphology, hemolytic pattern and Gram staining (Razmyar *et al*., 2013).

Preparation of cell lysates: From each plate which had single typical colony, one colony was suspended in 100 µl distilled water, boiled for 10 min and then centrifuged at 10,000 rpm for 10 min. The supernatants were collected carefully and used as template for PCR assay (Razmyar *et al*., 2013).

*Genus-specific PCR***:** The identity of the 25 recovered isolates was confirmed as *C. perfringens* based on the amplification of specific 16SrdNA gene, using specific primers (Table 1) (Wu *et al*., 2009).

Toxintyping: Six pairs of Specific primers corresponding to each toxin were used to determine the presence of *cpa*, *cpb*, *iA*, *etx*, *cpe* (Meer and songer, 1997), and *cpb2* genes (Bueschel*et al*., 2003), using a multiplex PCR technique for all isolates (Table 1**)**. Two strains, CIP 106157 *C. perfringens* (cpa+,*cpe*+) and CIP 60.61 *C. perfringens* (*cpa*+, *cpb*+, *etx+, cpb2*+) obtained from Pasteur Institute Collection (CIP France) were used as positive controls.

Amplification reactions were carried out in 50µl volume, containing 5 µl 10 x PCR buffer, 5 mMdNTPs, 25 mM $MgCl_2$, 5U of *Taq*DNA polymerase, 0.5 mM of each *cpa*oligo, 0.36 mM of each *cpb*oligo, 0.36 mM of each *cpb2* oligo, 0.52 mM of each *iA*oligo, 0.44 mM of each *etx*oligo, 0.34 mM of each *cpe*oligo, and dH_2O. Ten µl of DNA template was added to the mixture. Amplification was programmed in a thermocycler (Techne TC,3000, England) as follow: 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min (Meer and Songer, 1997). The amplified products were then analyzed by electrophoresis in a1.5% agarose gel under UV transillumination.

Results

Bacterial isolation: In the present study, from 200 minced meat samples, total of 25 (12.5%) *C. perfringens* isolates were identified, using conventional culture method and confirmed using genus-specific PCR assay, based on the sequence of 16SrdNA gene fragments.

Toxin genotyping of isolates: PCR amplification yielded the expected products for the 16Srd NA gene (279bp), and 12.5% of minced meat samples were determined as contaminated with *C. perfringens*. Toxin typing of isolated *C. perfringens* showed that type A was the most predominant type (88%), while type B, D and E displayed the incidence of 4%, for each type.Amount of22 isolates (88%) were determined as type A, and 3 isolates were determined as type B (No=1(4%)), type C (No=1(4%)) and type E (No=1(4%)). From 22type A isolates, 18 isolates (81.8%) were determined as simple type A (carrying neither the *cpe*nor*cpb2* gene) and 4 (18.1%) isolates were determined as heterogeneous types(carrying *cpb2* gene) but none of the isolates were found to carry both the *cpb2* and *cpe* genes. cpb2 gene was found in 4 isolates of type A and in one isolate of type B(20%).

Discussion

Pilgrimage and tourist attractions of the Mashhad city in Northeastern of Iran, which is known as the World's second pilgrimage city after Mecca, and the amount of food which

Table 1. Primers sequence and product size used for species-specific PCR and multiplex PCR

Njjoo9	Primer sequences (5`-3`)	Product length (bp)
16S rdNA	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279 (Wu *et al*., 2009)
cpa	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324(Meer and songer 1997)
cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196(Meer and songer 1997)
etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655(Meer and songer 1997)
iA	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446(Meer and songer 1997)
cpe	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233(Meer and songer 1997)
cpb2	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	567(Bueschel et al., 2003)

Table 2. Results of Multiplex PCR genotyping of C.perfringensisolated from minced meat.

	Toxin genes of *c. perfringens*					
type	***Cpa***	***cpb***	***etx***	***ιA***	***cpe***	***Cpb2***
A	4(18%)	_	_	_	_	4(18%)
A	18(81%)	_	_	_	_	_
B	1(4 %)	1(4 %)	1(4 %)	_	_	1(4 %)
D	1(4 %)	_	1(4 %)	_	_	_
E	1(4 %)	_	_	1(4 %)	_	_
total	25(12.5%)	1(4 %)	2(8%)	1(4 %)	_	5(20%)

serves for these tourism population indicates *the importance of* attention that must be taken to food-borne diseases. Following *Campylobacter* and *Salmonella* Spp., *Clostridium perfringens* is the third most important bacterial food-borne pathogen (Novak and Juneja, 2002). Meat and meat products are excellent medium for growth of this bacterium (Wen and McClane, 2004). Foods like kebab which contain minced-meat have a high popularity in Middle East and are prepared with the application of low heat treatment. The quality of these foods depends on meat and additives quality, sanitary condition of the equipments and the processing procedures (Atwa and Abou EI-Roos, 2011).

Traditionally, typing of *C. perfringens* strains involved sero-neutralisation of culture filtrates and mice or guinea pigs injection with culture supernatants of *C. perfringens*, along with antitoxin, and death (mice) or dermonecrosis (guinea pigs) was assessed (Sterne, 1975). This assay was extremely time consuming, as growth of the organism was required. It was also expensive as two of the toxins, epsilon and iota, required trypsin for activation, but a third toxin, beta toxin, was inactivated by trypsin. Therefore each culture supernatant was assayed numerous times, with and without trypsin, and with and without the five different preparations of neutralizing antisera (Hatheway, 1990). Multiplex PCR assay is a useful alternative to traditional assays and as a replacement for standard *in vivo* typing methods (Meer and Songer, 1997).

In our study the contamination rate of minced meat samples with *C. perfringens,* was 12.5%. Higher incidence has been reported by other researchers who reported the incidence of *C. perfringens*in minced meat samples as 35% (Torkyand A. Sh, 2004) and 28% (Atwa and Abou EI-Roos, 2011). Guran *et al* (2014)

reported that 96 % of ground beef and 88 % of ground sheep meat samples as contaminated with *C. perfringens*.Wen and McClane (2004) showed that 23% of American ground beef were contaminated with *C. perfringens.*Kamber *et al* (2007) also reported *C. Perfringens* isolation from17% of minced meat samples, with12%, 1%, 4% and 2% astypes A, B, C and D, respectively. Variation in contamination rates reported by different researchers might be due to differences in the technology of slaughtering, personnel and equipment hygiene, number of collected samples and the method of bacterial isolation.The other possibility is that, the present study was conducted during a season with low rainfall, which may have resulted in a lower number of animals suffering from diseases induced by *C. perfringens* and thus, lower contamination of carcasses with this bacteria.

In our study *C. perfringens* types A (*cpa*positive), B (*cpa*, *cpb*and *etx* positive), D (*cpa* and *etx* positive) and E (*cpa* and *iap* positive) were identified in the samples, whereas type A was the dominant type (88%-22out of 25). To the best of our knowledge, the present study is the first investigation on *C. perfringens* genotyping, isolated from minced meat (beef and sheep) in Iran. Guran *et al* (2014) by using the same method for toxintyping of the isolates in their study, demonstrated that, 77.4 % of isolates possessed only the *cpa* gene (simple type A), while 7.6 % carried the *cpa* and *cpb2* toxin genes (type A-$cpb2^{+}$), and 2.2 % the *cpa* and *cpe* toxin genes (type A-cpe^{+}), furthermore they reported 1.5 %, 3.8 % and 7.2 %of samples as types B, C and D respectively which are approximately similar to our findings.

*C. perfringens*enterotoxin (*Cpe*) is the most important virulence factor when type A isolates cause human GI diseases, although less than 5% of all *C. perfringens* produce this toxin (Heikinheimo *et al.,* 2004). In this study, all isolates were identified as*cpe*-negative which may indicates that contamination during pre-processing is unlikely, but processing, cutting, handling, and wrapping operations may be responsible for the addition of *C. perfringens* spores and vegetative cells (Hall and Angelotti, 1965). In this study, 5 out of 25 (20%) of *C. perfringens* isolates were identified as positive for *cpb2* gene. *Cpb2* toxin can be produced by all types of *C. perfringens* (Fisher, 2006). Therefore the presence of such genes is not considered a risk by itself and there are some predisposing factors that have been associated with the pathogenicity of toxigenic *C.perfringens* and consequently the development of disease (Crespo *et al.,* 2007). In another study the researchers did not find any *cpb2* positive *C. perfringens* in ground beef samples (Wen and McClane, 2004). In contrast to our results that indicate the presence of *cpb2* gene in 4 isolates of type A and in one isolate of type B, until now all reported *cpb2* positive *C. perfringens* isolates from various foods were from type A (Guran *et al*., 2014). This study reports the first*cpb2* positive type B of *C. perfringens* in minced meat.

In conclusion, multiplex PCR assay provides a simpleand rapid method for genotyping of *C. perfringens*isolates. This study showed that type A strain of *C. perfringens*is the mostprevalent types in minced meat in this region of Iran.

Further investigations are required to reveal the pathogenicity of *C. perfringens* type B with *cpb2*gene.

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جداسازی و تایپینگ مولکولی باکتری کلستریدیوم پرفرینجنس جدا شده از گوشت چرخ کرده

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چکیده

هدف از این مطالعه تعیین ژن های توکسین زا در باکتری کلستریدیوم پرفرینجنس جدا شده از ۲۰۰ نمونه گوشت چرخ کـرده بـه روش تایپینگ مولکولی بود. پس از جداسازی کلستریدیوم پرفرینجنس به روش کشت سنتی و تایید تشخیص با استفاده از PCR ژن های 16S rDNA،ژن های آلفا، بتا، بتا ۲، اپسیلون، یوتا وانتروتوکسین با روش مولتی پلکس PCR ردیابی شدند. ازمجموع۲۰۰نمونه مورد بررسـی، کلستریدیوم پرفرینجنس از۲۵ نمونه(۱۲.۵٪)جداسازی گردید. نتایج حاصل از مولتی پلکس PCR نشان داد که ۸۸٪ نمونه هـا بـه عنـوان تیپ A ، ۴٪ تیپ B، ۴٪ تیپ D و ۴٪ ازنمونه ها به عنوان تیپ E مورد شناسائی قرار گرفت. در این بررسی ۷۲٪ از جدایه هـا (۱۸) تنهـا دارای ژن *cpa* و ۱۶ ٪ از جدایه ها(۴) دارای ژن *cpa* به همراه ژن *cpb2* بودند. همچنین نتایج به دست آمده نشان می دهد کـه تیـپ A رایج ترین تیپ کلستریدیوم پرفرینجنس در گوشت چرخ کرده در ایران می باشد.

واژگان کلیدی: کلستریدیوم پرفرنجنس،PCR چندگانه و گوشت چرخ کرده